

RESEARCH ARTICLE

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Transcriptional and post-transcriptional regulation of the jasmonate signalling pathway in response to abiotic and harvesting stress in *Hevea brasiliensis*

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Abstract

Background: Latex harvesting in *Hevea brasiliensis* amounts to strong abiotic stress that can cause a halt in production in the most susceptible clones. Although the role of jasmonic acid has been suggested in laticifer differentiation, its role in latex production and in the response to harvesting stress has received very little attention. Only a few key genes acting in the COI-JAZ-MYC module have been isolated and studied at transcriptional level.

Results: Use of a reference transcriptome obtained on rubber clone PB 260 covering a large number of tissues under different environmental conditions enabled us to identify 24 contigs implicated in the jasmonate signalling pathway in the rubber tree. An analysis of their expression profile by qPCR, combined with hierarchical clustering, suggested that the jasmonate signalling pathway is highly activated in laticifer cells and, more particularly, in the response to harvesting stress. By comparison with their genomic sequences, the existence of regulation by alternative splicing was discovered for JAZ transcripts in response to harvesting stress. Lastly, positive transcriptional regulation of the *HbJAZ_1405* gene by MYC was demonstrated.

Conclusion: This study led to the identification of all actors of jasmonate signalling pathway and revealed a specific gene expression pattern in latex cells. In-depth analysis of this regulation showed alternative splicing that has been previously shown in *Arabidopsis*. Interestingly, genotypic variation was observed in *Hevea* clones with contrasting latex metabolism. This result suggests an involvement of jasmonate signalling pathway in latex production. The data suggest that specific variability of the JA pathway may have some major consequences for resistance to stress. The data support the hypothesis that a better understanding of transcriptional regulations of jasmonate pathway during harvesting stress, along with the use of genotypic diversity in response to such stress, can be used to improve resistance to stress and rubber production in *Hevea*.

Keywords: Latex, Tapping panel dryness, Jasmonic acid, Alternative splicing, Rubber, Transcriptional regulation

Background

Jasmonic acid (JA) is a key hormone in the development of plant responses to abiotic stress, such as wounding [1]. This plant hormone also plays a key role in the biosynthesis of secondary metabolites [2]. It is particularly involved in the response to oxidative stress since it activates the ascorbate-glutathione cycle for the reduction of these major antioxidants [3,4].

The jasmonate biosynthesis and signalling pathways have been very widely studied and described in *Arabidopsis* using the analysis of mutants [5]. The *jar1* mutant is deficient in certain responses to JA, including ascorbate production to prevent oxidative stress [6]. The JAR1 enzyme catalyses the conjugation of jasmonate to isoleucine (JA-Ile) [7]. JA-Ile is the bioactive form of JA [8]. It was demonstrated recently that COI1 is the JA receptor and can interact directly with JA-Ile [9]. For its part, the *coi1* mutant also shows insensitivity to JA, thereby causing male sterility, resistance to the inhibition of root development by JA, and a defect in the expression of genes regulated by JA

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[10]. COI1 is an F-box protein [11] forming, with some other partners, a complex of the E3 ubiquitin ligase type, SCF^{COI1} [12,13]. This type of complex is involved in the ubiquitination of target proteins, leading to their subsequent degradation by the 26S proteasome [14]. JASMONATE ZIM DOMAIN (JAZ) proteins are recognized by the SCF^{COI1} complex. JAZ degradation enables the release of transcription factors such as MYC2,3 and 4 [15]. The latter bind to *cis*-acting elements of the G-box type [16] present in the promoters of JA response genes and they initiate their transcription [17-19]. In addition to the MYC transcription factors, JAZ are able to interact with other transcription factors of the MYB, EIN3, EIL, ERF, GAI, RGA and RGL1 types, suggesting that they play a role in the interaction of the JA signalling pathway with those of other hormones [20,21]. In *Arabidopsis*, JAZ proteins comprise 12 members which are characterized by the presence in the C-terminal position of the highly conserved domains, Jas and ZIM (TIFY). The Jas domain enables interaction with COI1 and with transcription factors, while the TIFY domains involved in dimerization and in the interaction with NINJA [21-23]. At the moment, the most likely model concerning the repression of genes induced by JA is an interaction of JAZ with TOPLESS (TPL). This interaction may necessitate the presence of the NINJA protein as an adapter, unless the JAZ protein possesses an EAR domain (ERF-associated amphiphilic repressor) to which TPL binds [1]. The transcriptional regulation of JAZ can occur via MYC2 [16], but other components might be involved. Indeed, in the *myc2* mutant, most JAZ were expressed following infection with *Pseudomonas syringae* [24]. The sub-unit of the mediator complex, MEDIATOR25 (MED25), was recently identified as an integrative node of JA-dependent gene expression [25]. At post-transcriptional level, most JAZ genes can be regulated by alternative splicing. In *Arabidopsis*, it has been found that intron/exon organization in the region of the Jas domain is conserved in the majority of JAZ genes [26]. In *Arabidopsis*, the spliced form of JAZ10 has a Jas domain that is partially (JAZ10.3) or totally truncated (JAZ10.4) [23]. Ectopic expression of JAZ10.3 and JAZ10.4 affords dominant insensitivity to JA as a consequence of the stabilization of the JAZ protein [19,23]. The physiological role of isoforms with a truncated Jas domain in attenuating hormonal response has been suggested by various studies [19,27]. Many studies have shown the importance of alternative splicing in plant development, but also in the response to stress (for review [28]), though that link has never been demonstrated in the case of post-transcriptional regulation of JAZ genes.

Hevea brasiliensis is the only commercial source of natural rubber (NR). NR is synthesized in the rubber particles of latex cells. Those cells differentiate from the cambium then anastomose to form an articulated network: laticifers.

Latex is harvested by tapping the tender bark (phloem tissues). After latex flow, rubber particles agglomerate and clog the tapping zone. Latex is regenerated within a few days. In order to stimulate latex production 2.5% ethephon (an ethylene releaser) is applied to the tapping panel. Tapping and stimulation frequency is usually adapted to the metabolic activity of *Hevea* clones. In extreme cases of environmental or man-made stress due to rubber tree tapping, an oxidative burst occurs in the laticifers. That oxidative stress leads to peroxidation of lipids in the membrane of luteoids (poly dispersed lysosomal vacuole), which contain agglutinins such as heveins. The release of these factors leads to the *in situ* coagulation of rubber particles: this is the physiological syndrome known as Tapping Panel Dryness (TPD) [29]. TPD causes substantial NR production losses (10-40%) and economic models predict that NR production will not be sufficient by 2020. The genetic variability existing within the different cultivated rubber clones shows that the intrinsically most productive and fast growing clones are also the most susceptible to abiotic stress and TPD [30]. Some rubber clones with a slow laticifer metabolism are more tolerant of TPD. To date, few selected clones have the latter characteristics [31]. For instance, clone PB260 is a clone with an active metabolism that is highly susceptible to TPD, while INC53 and RRIM600 are clones with a slower metabolism and are more resistant to TPD. The physical wounding caused by tapping, the osmotic shock within the laticifers due to latex flow, and metabolic activation linked to latex regeneration between two tapplings amount to harvesting stress which causes diverse responses, including the production of hormones such as JA. In fact, wounded tissues produce systemin, which induces JA production [32]. Interestingly, jasmonate and wounding are also involved in laticifer differentiation [33]. It was recently shown that jasmonate acts as a signal molecule in rubber biosynthesis [34]. Although the jasmonate signalling pathway has been studied in the rubber tree in connection with rubber biosynthesis and laticifer differentiation, little is known about its role in the response to harvesting stress. To date, only the ethylene biosynthesis and signalling pathways have been largely studied in this connection, and notably the transcriptional regulation of *ERFs* (Ethylene Response Factor) [35-39]. In *Hevea*, one or two members of the multigene families encoding COI [40], JAZ [41] and MYC [42] have been identified. Studies on the expression profile of those genes suggest the importance of JA in latex production. Indeed, *HbCOI1* is strongly expressed in laticifers [40], the transcripts of *HbJAZ1* accumulate in response to tapping and wounding [41], and *HbMYC1* and *HbMYC2* are more abundant in latex. *HbMYC1* is induced by regular tapping and wounding, while the expression of *HbMYC2* is not altered by those stimuli [42].

In this study, the availability of *Hevea* transcriptomic and genomic resources made it possible to identify all the different genes acting in the jasmonate signalling pathway and characterize their implication during development, and in response to abiotic stress. Among the transcriptomes sequenced on different tissues (latex, bark, leaves and stem tips) [43-48], a reference transcriptome covering a large number of tissues and environmental conditions has been published for rubber clone PB 260 [35]. An analysis of the structure of *JAZs* genes based on the genomic sequences of rubber clone CATAS 7-33-97 (BIG-CATAS Project, unpublished data), and an analysis of the corresponding transcripts revealed the existence of a mechanism of alternative splicing of *JAZ* gene transcripts. Thus, this study suggested that the stress caused transcriptionally and post-transcriptionally by latex harvesting regulates the jasmonate signalling pathway in *Hevea*.

Methods

Plant material

For the transcript analyses, plant material of clone PB 260 grew in accordance with the conditions described in Duan and coll. [35]. Samples corresponding to reproductive tissues (immature and mature, male and female flowers, zygotic embryos) were added to this study. Flowers were collected from 15-year-old trees. Zygotic embryos were collected from 5-year-old trees. *In vitro* plantlets of clone PB 260 were obtained by somatic embryogenesis with line CI07060. The plantlets were acclimatized and grown for 3 months in a greenhouse at a temperature of 27°C with 45% humidity. Several treatments mimicking abiotic stress were carried out, such as wounding, methyl jasmonate (MeJA), ethylene (ET) and dehydration at 1, 4, 8 and 24 hours. Drought stress response was controlled by following *HbERFIVa3* (orthologue to DREB2A) transcript accumulation during this stress [49], whereas efficiency of wounding, ethylene and MeJA treatment were controlled using *HbERF-IXc4* and *HbERF-IXc5* (orthologue to ERF1) [37,50]. The bark was wounded every 0.5 cm by scarification with a razor blade. The ET and MeJA treatments were carried out by placing the plants in a 300-l open-door Plexiglas box overnight before treatment. Five parts per million of pure ET gas (1.5 ml/300 l) was injected into the sealed air-tight box. A concentration of 100 µl of liquid ≥95% MeJA solution (Sigma, St Louis, MO) was diluted in 500 µl of absolute ethanol and then placed on Whatmann paper inside the box for gas release. Control plants used for the ET and MeJA treatments were placed in the box and exposed to air only. The dehydration treatment was carried out by taking the plants out of their pots and leaving them to dry under laminar air flow. Each sample was collected 1 h, 4 h, 8 h and 24 h after treatment.

RNA samples were collected and prepared at the CRRC, RRIT, Sanam Chaikhet District, Chachoengsao 24160,

Thailand (13.39N latitude and 101.26E longitude). These locations and our activities did not require any specific permission. The field studies did not involve endangered or protected species.

Total RNA extraction

Total RNAs were extracted from one gram of fresh matter using the caesium chloride (CsCl) cushion method adapted from Sambrook and coll. (Sambrook et al. [51]) by Duan and coll. (Duan et al. [37]). DNA contamination was checked by PCR amplification using primers of the actin gene.

Primer design and transcript abundance analysis by qPCR

Total RNA integrity was checked by electrophoresis. For each candidate gene a blast was performed against the transcriptome of PB 260, to define highly conserved regions. The qPCR primers were designed outside these regions using the Primer 3 module of Geneious Pro software version 5.3.6 (Biomatters Ltd., New Zealand). Each primer pair was blasted against the transcriptome library. qPCR and the fusion curve of the PCR amplicon were done using a mix of cDNAs. In addition, the specificity of each primer pair was checked by sequencing the PCR amplicon. The sequences of the primers used are listed in Additional file 1.

cDNAs were synthesized from 2 µg of total RNA to a final reaction volume of 20 µl using a Revert Aid™ M-MuLV Reverse Transcriptase (RT) kit according to the manufacturer's instructions (MBI, Fermentas, Canada). Full-length cDNA synthesis was checked for each sample by PCR amplification of the actin. Quantitative gene expression analysis was carried out by qPCR using a Light Cycler 480 (Roche, Switzerland) as described by Putranto and coll. [52]. Real-time PCR was carried out for eleven housekeeping genes in order to select the most stable gene as the internal control for all the compared tissues (*HbELF1Aa*, *HbUBC4*, *HbUBC2b*, *HbYLS8*, *HbRH2b*, *HbRH8*, *HbUBC2a*, *HbalphaTub*, *Hb40S*, *HbUbi*, *HbActin*). *HbRH2b* was selected as the best reference gene due to its stability in the different tested tissues [39] (Additional files 2 and 3). The transcript abundance of each gene was relatively quantified by normalization relative to the transcript abundance of the *HbRH2b* reference gene. All the normalized ratios corresponding to transcript accumulation were calculated automatically by Light Cycler software version 1.5.0 provided by the manufacturer. Heatmap representation was carried out on normalized and centered ΔC_t values, using the heatmap2 function of the R software gplots package [53,54].

Statistical analyses

qPCR reactions were carried out with 3 biological replicates. The statistical analyses were ANOVAs carried out

on raw data after logarithmic transformation. An ad-hoc Tukey HSD (Honestly Significantly Different) test was carried out for the analysis of transcripts in the different organs and different clones. Values with the same letter were not significantly different. For the analysis of transcripts in response to abiotic stress and to hormone treatments, a comparison of means test (Student or Wilcoxon test, depending on the data) was carried out between the control and the treatment at each point of the kinetics. If, at one point, at least one significant difference was found an *s* was indicated, otherwise *ns* was indicated.

Phylogenetic analysis of JAZ genes

Multiple alignment was carried out on the total protein sequences of the JAZs of *Arabidopsis* and *Hevea*. Alignment with Gblocks software [55] led to the isolation of conserved blocks at least 10 amino acids long. The blocks were then used to construct the phylogenetic tree using PhyML software [56] which implements a maximum likelihood tree reconstruction method using the LG + gamma model, starting from a BioNJ tree [57]. A RAP-Green analysis was carried out using the original tree from PhyML to predict duplications [58]. The final tree was visualized with the Archaeopteryx program [59].

Test of transcriptional activity by transient expression in BY-2 cells of tobacco

Transactivation experiments were carried out following the procedure described by Chaabouni and coll. [60]. The *pJAZ_1405(-267)* and *pJAZ_1405(-955)* promoters were cloned to the pMDC107 vector [61], thereby controlling the GFP reporter gene. The *MYC_771* and *MYC_94937* genes were cloned under control of the 35S promoter to the pMDC32 vector. Transactivation assay was carried with several controls. First, a window excluding debris and define auto-fluorescence was defined from protoplast solution without transformation. Second, a negative control was obtained by protoplast transformation with the empty reporter vector and effector vector. Third, protoplast transformation with two vectors pJAZ and pHbMYC was carried out. The negative control is used as reference for the calculation of transactivation activity as follows: $(pJAZ_{1405(-955)} + pHbMYC) / (pJAZ_{1405(-267)} + pHbMYC)$. This method avoids any risk of disturbance due to the gateway cassette present in pMDC32 vector. The primer sequences used for gateway cloning are listed in Additional file 4. Transformation was replicated 6 times independently. After 16 h, GFP expression was quantified by flow cytometry (FACS Calibur II instrument, BD Biosciences) on the Montpellier Rio Imaging (MRI) platform. Data were analysed using Flowing Software version 2.5.0.

Accession numbers

Reads from this library are those published by Duan and coll. (Accession: PRJNA235297 ID: 235297) available on NCBI database.

Results

Identification of the different genes acting in the jasmonate signalling pathway

The different genes acting in the jasmonate signalling pathway were identified from the comprehensive transcriptome published by Duan and coll. [35] using TBLASTN with the corresponding *Arabidopsis* sequences as the query. For each gene, we selected the one that came out with the best score. For each gene identified in that way, we checked for the conservation of the domains characteristic of each family using INTERPROSCAN. We thus identified 6 contigs corresponding to *JAR*, 2 for *COI*, 10 for *JAZ* and 3 for *MYC*. The *JARs* belonged to the GH3 multigene family [62]. GH3s are generally characterized by the presence of 3 small conserved motifs [63]. Motif III was found to be highly conserved in the 6 *Hevea* sequences. Motifs I and II were found in *JAR_5108*, *JAR_14894* and *JAR_59958*, while *JAR_20347* and *JAR_20244* did not display motif I. The absence of motif I in the N terminal part of the proteins deduced from the *JAR_20347*, *JAR_21367* and *JAR_20244* contigs was linked to the fact that the contig sequences were incomplete at 5' (Additional file 5). An analysis of the protein sequences revealed that the two HbCOI, HbCOI_2304 and HbCOI_3058, were characterized by the presence of an F-box domain, along with 3 leucine-rich repeat (LRR) domains (Additional file 6). All of the JAZ proteins identified had the 2 characteristic domains, TIFY and Jas (Additional file 7) [64]. HbMYC_424, HbMYC_771 and HbMYC_94937 all had a bHLH DNA binding domain, along with the 4 conserved regions (I to IV) (Additional file 8) [65]. An analysis of the transcriptome of rubber clone PB260 also enabled us to identify the partners *HbNINJA_6328* (Additional file 9) and *HbTPL_7591* (Additional file 10). HbNINJA displayed 46% of identical residues with At4g28910, while HbTPL displayed very strong homology with At1g15750 as 85% of the amino acids were identical. We were also able to identify an orthologue of AtMED25, HbMED25_16787, which displayed 50% identity (Additional file 11).

Expression profile of the different genes acting in different developing tissues

In order to characterize the JA signalling pathway in different *Hevea* tissues, the transcript abundance of all the genes identified was analysed by qPCR in zygotic embryos (embryo body and cotyledon), roots (TR: taproot and SLR:secondary lateral root), bark, leaves, latex, and male and female flowers at the immature and mature stages (Figure 1). Heatmap representation combined with

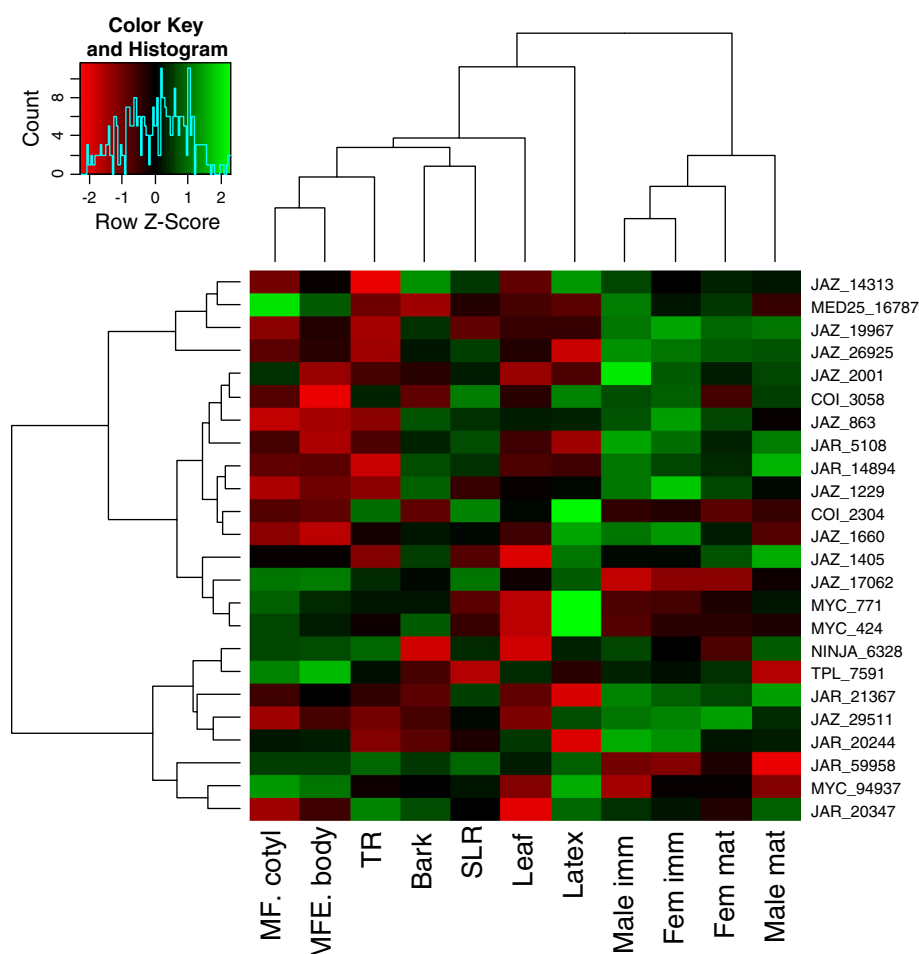


Figure 1 Heatmap representation of the expression of genes acting in the jasmonate pathway in different rubber tree tissues. The data obtained by quantitative RT-PCR corresponded to the levels of the actor transcripts in total RNA samples extracted from mature fruit cotyledon (MF. cotyl), mature fruit embryo bodies (MFE.body), taproots (TR), bark, secondary lateral roots (SLR), leaves, latex, immature male flowers (Male imm), immature female flowers (Fem imm), mature female flowers (Fem mat), and mature male flowers (Male mat). The data presented correspond to averages of 3 independent biological replicates. The red and green colours correspond to low and high gene expression, respectively. The heat map was generated using R software.

an analysis of variance (ANOVA) was used to class the genes according to their level of expression in each tissue (Additional file 12). In addition, hierarchical clustering was used to identify tissues that displayed the same transcriptional signature. The different parts of the embryo displayed the same transcriptional landscape. Overall the jasmonate-related genes implicated in flowers were those that were not expressed in the embryo. Male and female flowers, whether mature or immature, were grouped together, suggesting that the same jasmonate-related genes were involved, whatever the flower gender and development stage. The transcriptional signature in the taproot (TR) was grouped with the embryo, while most of the genes expressed in the secondary lateral roots (SLR) were also expressed in bark. Surprisingly, latex displayed an original profile, with a large number of over-expressed signalling genes (*JAZ_14313*, *COI_3058*, *COI_2304*, *JAZ_1660*,

JAZ_1405, *JAZ_17062*, *MYC_771*, *MYC_424*, *MYC_85795*, *MYC_94937*). These results confirmed the importance of the jasmonate pathway in latex. In addition, it was interesting to see that *TPL_7591* and *NINJA_6328*, which, *a priori* function together, were in the same cluster. *MED25_16787* had an expression profile similar to that of *JAZ_14313*.

Regulation by abiotic stress of genes acting in the jasmonate pathway

The relative transcript abundance was monitored over time for the 24 contigs corresponding to the genes of the jasmonate signalling pathway in response to wounding, dehydration, ethylene and methyl jasmonate (MeJA) (Figure 2). In the aim to validate efficiency of treatments, *Hevea* orthologous of *Arabidopsis* genes, known to be regulated by wounding, dehydration, ethylene and MeJA were used as positive control. Orthologous to DREB2 and ERF1

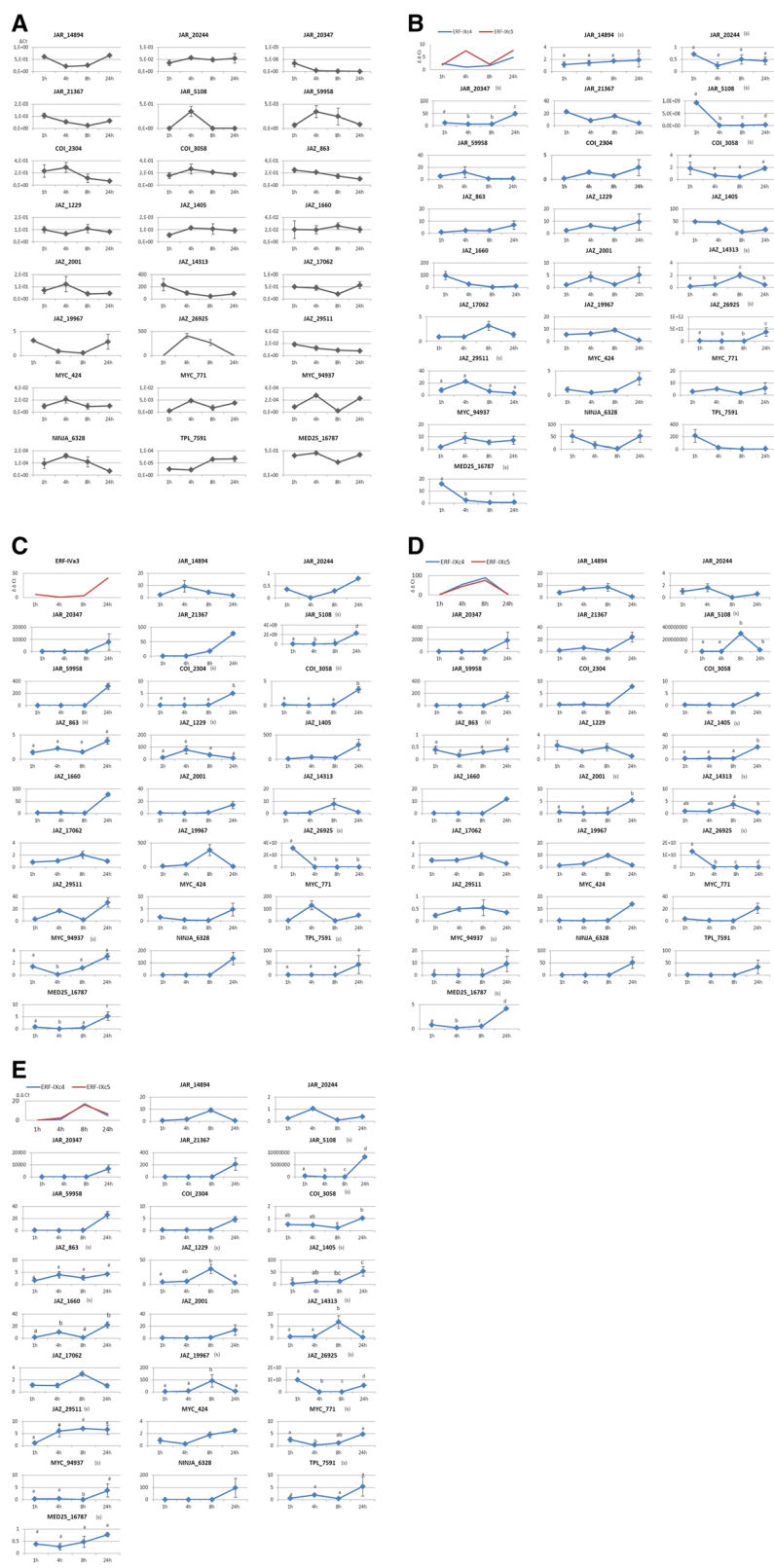


Figure 2 (See legend on next page.)

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Figure 2 Expression pattern of the jasmonate transduction pathway members in *Hevea* plantlets in response to abiotic and hormonal stress. The data obtained by quantitative RT-PCR corresponded to the transcript accumulation of genes acting in the JA signalling pathway in response to wounding, dehydration, ethylene and methyl jasmonate (MeJA) in stems of 3-month-old plantlets. RNAs were collected after 1 h, 4 h, 8 h and 24 h of treatment. Gene expression values were normalized using *Rh2B* as internal control, and then, ratio between treated and untreated plant have been calculated for each time point. A comparison of means test was carried out to compare the transcript abundance in stressed plants and control plants. Significant changes were indicated by * above error bars. **(A)** Expression pattern of jasmonate pathway genes in control plant. **(B)** Expression pattern of jasmonate pathway genes in response to wounding. Expression pattern of *HbERF1* and *HbERF2* have been tested to validate efficiency of wounding treatment. **(C)** Expression pattern of jasmonate pathway genes in response to dehydration. Expression pattern of *HbERF1* has been tested to validate efficiency dehydration treatment. **(D)** Expression pattern of jasmonate pathway genes in response to ethylene. Expression pattern of *HbERF1* and *HbERF2* have been tested to validate efficiency ethylene treatment. **(E)** Expression pattern of jasmonate pathway genes in response to jasmonate. Expression pattern of *HbERF1* and *HbERF2* have been followed to validate efficiency jasmonate treatment. The data presented correspond to the mean of 3 independent biological replicates.

were identified in *Hevea* by Piyatrakul and coll. [37]. Expression of the *DREB2* genes is induced by drought stress and is involved in drought-responsive gene expression [49]. *HbERF-IVa3* is an orthologous of *DREB2A* and *DREB2B* [37]. *ERF1* is induced by various stresses including wounding and hormonal treatments, acts at the crosstalk of jasmonate and ethylene signalling pathways. Its two putative orthologous genes in *Hevea*, *HbERF-IXc4* and *HbERF-IXc5* [37], were used to validate wounding, ethylene and jasmonate treatments in this study. The transcript abundance of all the genes of the jasmonate signalling pathway was modified between 1 h and 24 h, whatever the treatment. The transcripts of *JAR_14894*, *JAR_20347*, *JAR_5108* accumulated significantly in response to wounding, while the transcript abundance of *JAR_20244* was significantly lower than in the control (Figure 2A,B). Only the transcript of *JAR_5108* accumulated significantly in response to dehydration. For each type of stress, at least one transcript of each partner of the COI-JAZ-MYC module was highly activated (Figure 2C). Interestingly, several genes acting in the signalling pathway were also regulated by ethylene (Figure 2D). Statistical analyses showed that *JAR_5108*, *JAZ_863*, *JAZ_1405*, *JAZ_2001*, *JAZ_14313*, *JAZ_26925* and *MYC_94937* were regulated by ethylene. It should be noted that, among the *JAR* and *COI*, only the transcripts of *JAR_5108* and *COI_3058* were significantly regulated by JA (Figure 2E). Of the 10 *JAZ* genes studied, the transcripts of eight of them were significantly accumulated in response to JA (*JAZ_863*, *JAZ_1229*, *JAZ_1405*, *JAZ_1660*, *JAZ_14313*, *JAZ_19967*, *JAZ_26925*, *JAZ_29511*). The transcripts of *MYC_771* and *MYC_94937* were significantly accumulated in response to JA. *TPL_7591*, *NINJA_6328* and *MED25_16787* were regulated overall in the same manner. In response to wounding, their transcript abundance increased rapidly in 1 h, while in response to ET and MeJA, they only accumulated after 24 h. Taken together, these results suggest that the genes involved in JA signalling are expressed in response to abiotic stress, but it was not

always the same *JARs*, *COIs*, *JAZs* and *MYCs* that were involved depending on the stress in that signalling.

Genotypic regulation of genes involved in the JA pathway in response to harvesting stress in mature trees

The transcript abundance of genes belonging to the COI-JAZ-MYC module were studied in the latex and bark of tapped rubber trees (Figure 3, Additional file 13). During harvesting, the transcripts of the COI-JAZ-MYC module were regulated in both latex and bark. Our results showed that the expression level for *COI_2304* was significantly higher in latex than in bark ($p < 0.01$). In latex, the transcripts of *COI_2304* were accumulated in response to tapping, while in bark the transcript abundance decreased in response to that stress ($p < 0.01$). Treatment with ethephon tended to reduce the transcript abundance of *COI_3058* ($p < 0.01$) in latex and in bark. The results suggested that the transcript regulation of *JAZ* genes was specific to each gene and that no general tendency could be brought out for the family as a whole. For example, *JAZ_1229*, *JAZ_2001* and *JAZ_19967* were significantly more expressed in bark than in latex, while the reverse was true for *JAZ_1405*, *JAZ_1660*, *JAZ_17062* and *JAZ_29511*. In latex, *JAZ_1405* was the only *JAZ* to be significantly repressed by tapping ($p < 0.01$) (Additional file 13), while *JAZ_863* ($p < 0.01$), *JAZ_1660* ($p < 0.01$), *JAZ_17062* ($p < 0.01$), *JAZ_29511* ($p < 0.01$) and *JAZ_26925* ($p < 0.01$) were induced by tapping when there was no ethephon treatment. Interestingly, the transcripts of *JAZ_17062* and *JAZ_26925* were accumulated in an opposite manner in latex and bark in response to tapping. In bark, *JAZ_863* ($p < 0.01$), *JAZ_1229* ($p < 0.01$), *JAZ_19967* ($p < 0.01$), *JAZ_1660* ($p < 0.01$), and *JAZ_29511* ($p < 0.01$) were induced by tapping, while the expression of *JAZ_2001* was repressed by tapping ($p < 0.01$). In the majority of cases, ethephon reduced *JAZ* transcript abundance. However, in latex, the transcripts of *JAZ_29511* and *JAZ_863* were accumulated in the presence of ethephon ($p < 0.01$ and $p < 0.01$, respectively). In bark, when the tree was

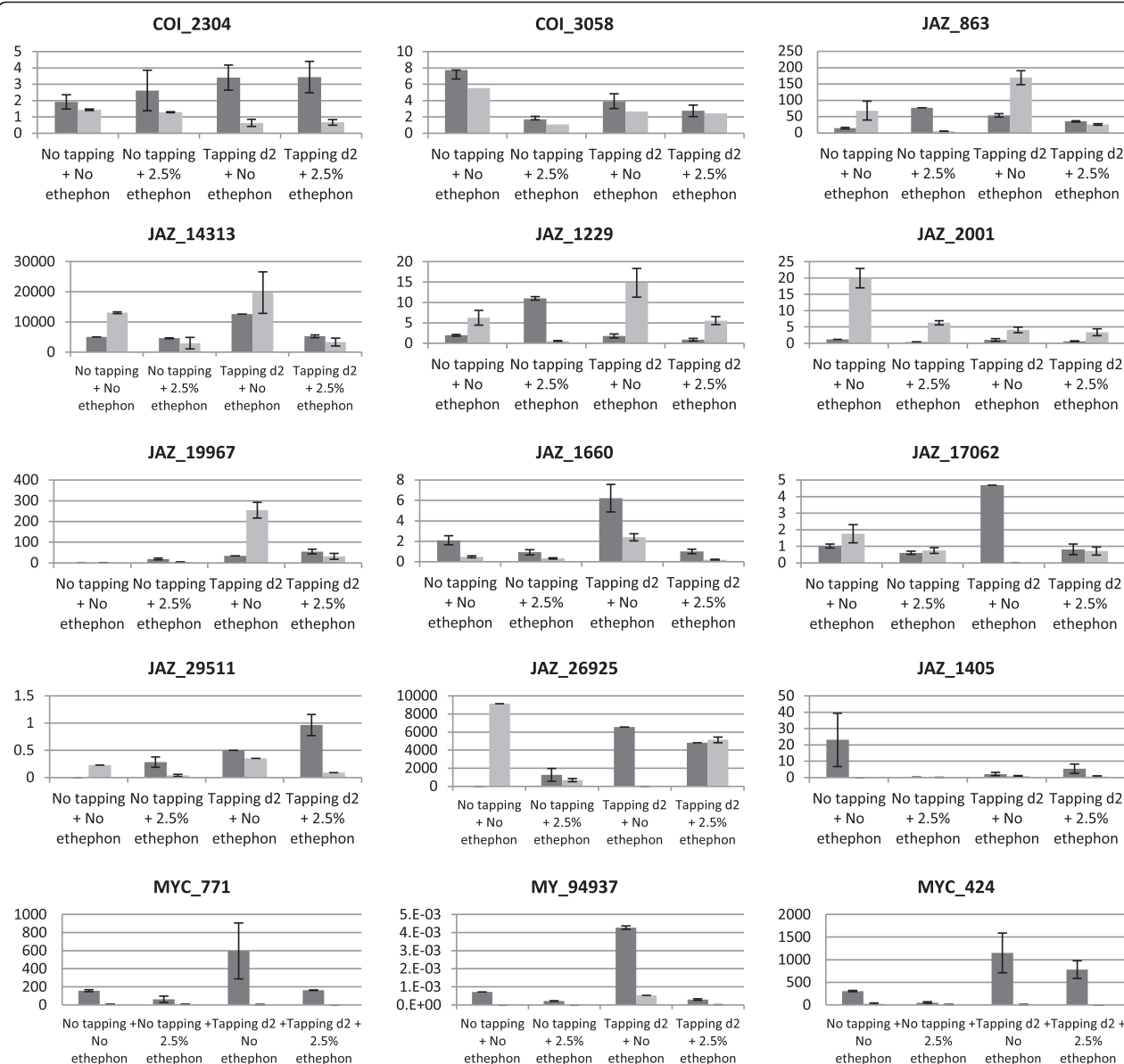


Figure 3 Regulation of COI-JAZ-MYC module members in *Hevea* in response to harvesting stress in latex and in bark. The accumulation of each COI-JAZ-MYC member was studied in the latex (dark grey bar) and bark (light grey bar) of adult trees, during latex production. During latex production the trees were tapped (3,4) every 2 days, or not (1,2), and stimulated by 2.5% ethephon, once per month (2,4), or not (1,3). The data presented correspond to averages of 3 independent biological replicates.

tapped, the transcripts of *JAZ_26925* were accumulated in the presence of ethephon ($p < 0.01$). The 3 MYC factors tested displayed the same expression profile. In fact, they were expressed more in latex than in bark and were strongly induced by tapping. Together, these results suggested that JA played a role during latex harvesting.

Clonal regulation of genes acting in the jasmonate signalling pathway

In order to find out whether the jasmonate signalling pathway might be involved in the differences in laticifer

metabolism observed at clonal level, we studied the transcript abundance of each of the genes acting in the pathway in clones PB260, INC53 and RRIM600. Of the 24 genes studied, only 8 displayed a transcript abundance that was significantly different between clones (Figure 4, Additional file 14). *JAR_59958* and *MYC_771*, positive regulators of the JA signalling pathway, were highly expressed in INC53 and RRIM600. On the other hand, the transcripts of the JAZ negative regulators were more expressed in clone PB260. These results showed that the positive regulators of the JA pathway are preferentially accumulated in

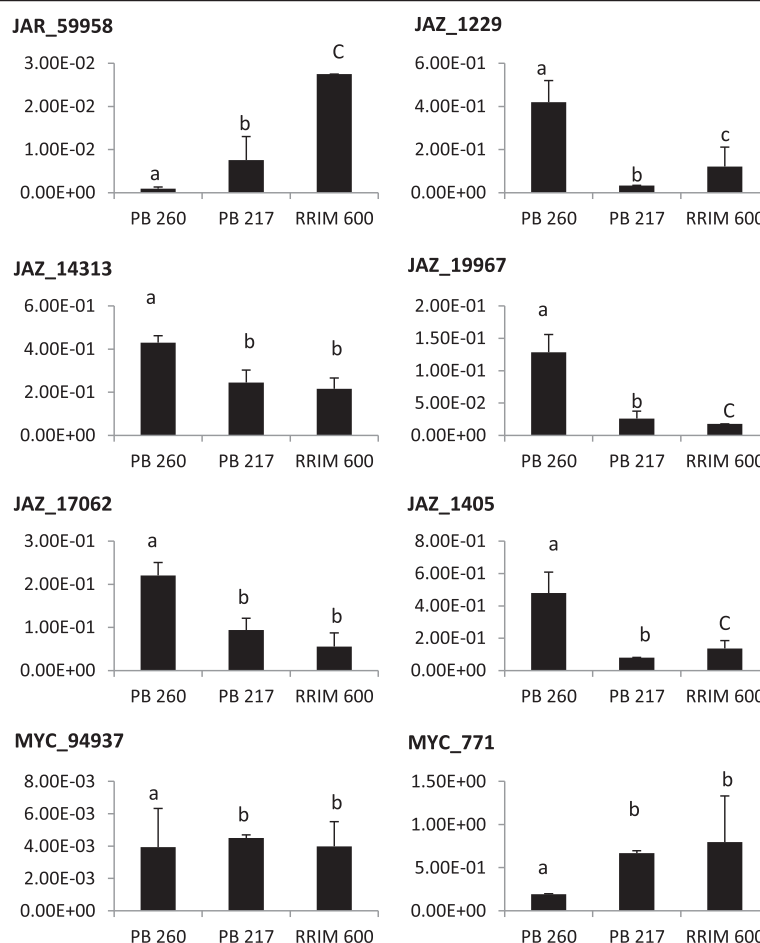


Figure 4 Expression of genes acting in the jasmonate pathway in 3 *Hevea* clones. The transcript abundance of the 24 identified genes was studied by qPCR on latex from PB 260, INC 53 and RRIM 600. According to an analysis of variance 8 transcripts were significantly over-represented in one or two clones (Additional file 14). The data presented correspond to averages of 3 independent biological replicates. Different letters correspond to significantly different values after the Tukey HSD test ($p < 0.05$).

clones with a slow laticifer metabolism, while the negative regulators are accumulated more in clones with a rapid laticifer metabolism.

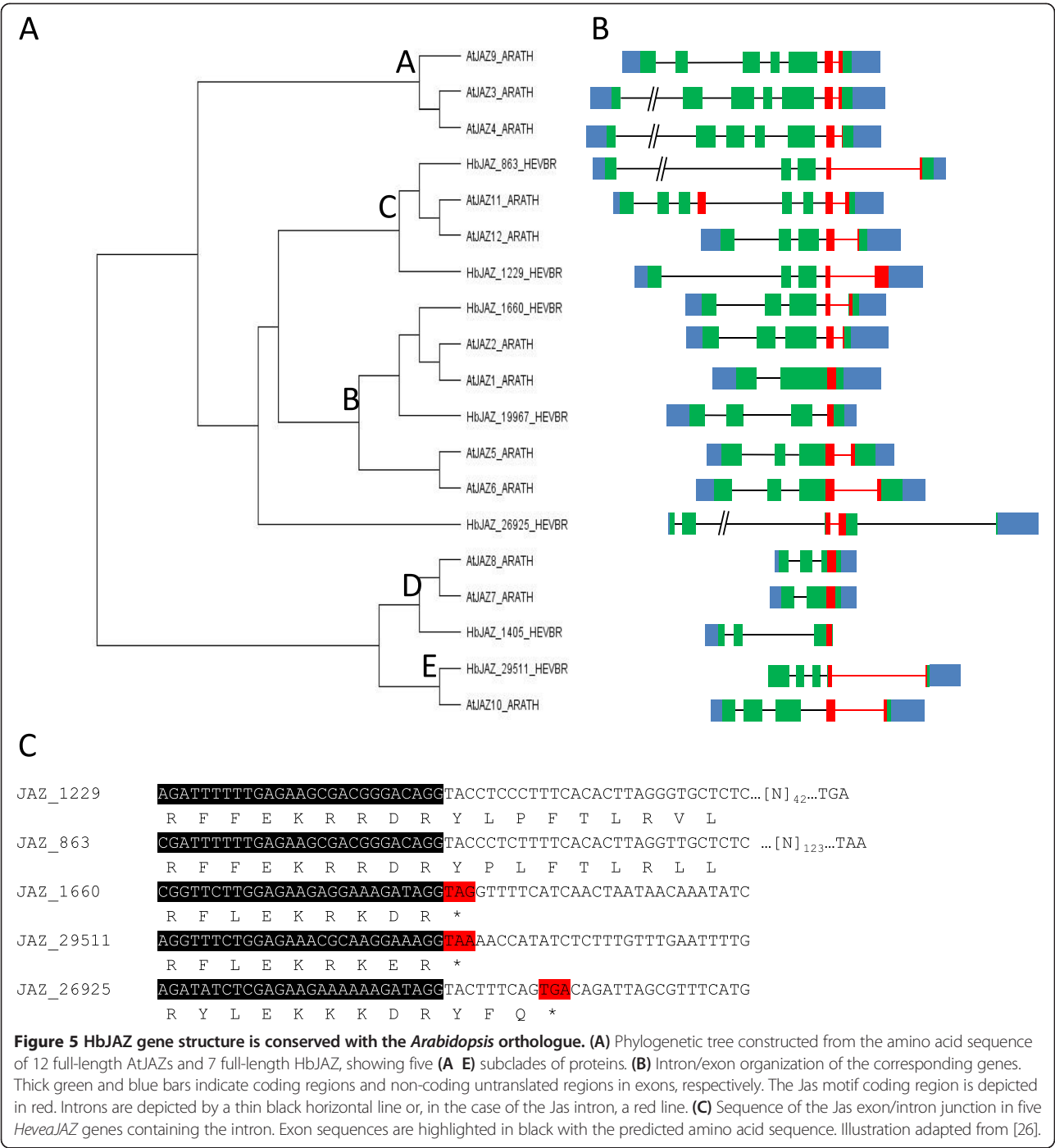
Conservation of the structure *HbJAZ* genes and that of their *Arabidopsis* orthologue

A study of the phylogeny and structure of the *JAZs* of *Hevea* was carried out on 7 *JAZs* (*JAZ_1660*, *JAZ_19967*, *JAZ_1405*, *JAZ_29511*, *JAZ_26925*, *JAZ_863*, *JAZ_1229*) which were of interest because they are regulated both by JA and by tapping. A phylogenetic analysis based on the protein sequences of the *HbJAZ* and *AtJAZs* revealed an organization primarily in three sub-classes B, C and D previously defined by Chung [26], with sub-class A not being represented by any *JAZ* member of *Hevea* (Figure 5A). *AtJAZ10*, previously assigned to sub-class C, revealed stronger homology with *HbJAZ_29511* to form a new sub-class that we called sub-class E. *HbJAZ_26925* was not grouped in the previously described sub-classes and

formed a new sub-class. The gene structure of the 7 *JAZs* of interest was analysed using the scaffold sequences corresponding to the genome of rubber clone CATAS 7-33-97 (Figure 5B). Most rubber tree *JAZs* possess an intron in the Jas domain, which is similar to what has been described in *Arabidopsis*. The sequence analysis predicted that retention of the 5' region of the Jas intron splicing site during pre-mRNA processing of *JAZ_1660*, *JAZ_29511* and *JAZ_26925* generated a premature termination codon immediately or very rapidly (Figure 5C). By using both primers overlapping the splicing zone and primers inside the intron, it was possible to confirm alternative splicing by sequencing of the qPCR amplicons for *JAZ_1229*, *JAZ_863*, *JAZ_1660*, *JAZ_29511* and *JAZ_26925*.

Alternative splicing of *HbJAZ* is regulated by tapping stress

In order to gain a clearer understanding of *JAZ* post-transcriptional regulation in response to tapping stress in rubber trees, we used qPCR to measure the spliced



form/non-spliced form ratio of transcripts in the latex and bark of tapped or untapped rubber trees, treated or not with ethephon (Figure 6, Additional file 15). Despite the existence of non-spliced forms for the transcripts of *JAZ_29511*, *JAZ_863* and *JAZ_26925*, this was not detected by qPCR in the samples tested. The alternative splicing of *JAZ_1229* and *JAZ_1660* was significantly regulated by tapping and by ethephon treatment. Our results suggested opposite regulation between the splicing of

JAZ_1229 and *JAZ_1660*. In fact, tapping induced the non-spliced form of *JAZ_1229*, suggesting an increase in the repressive form of that *JAZ*, while the same stress induced the spliced form of *JAZ_1660*.

MYC_771 and MYC_94937 regulate the transcription of *JAZ_1405*

The transcripts of certain *JAZs* were not subjected to alternative splicing like *JAZ_1405*. Hierarchical clustering

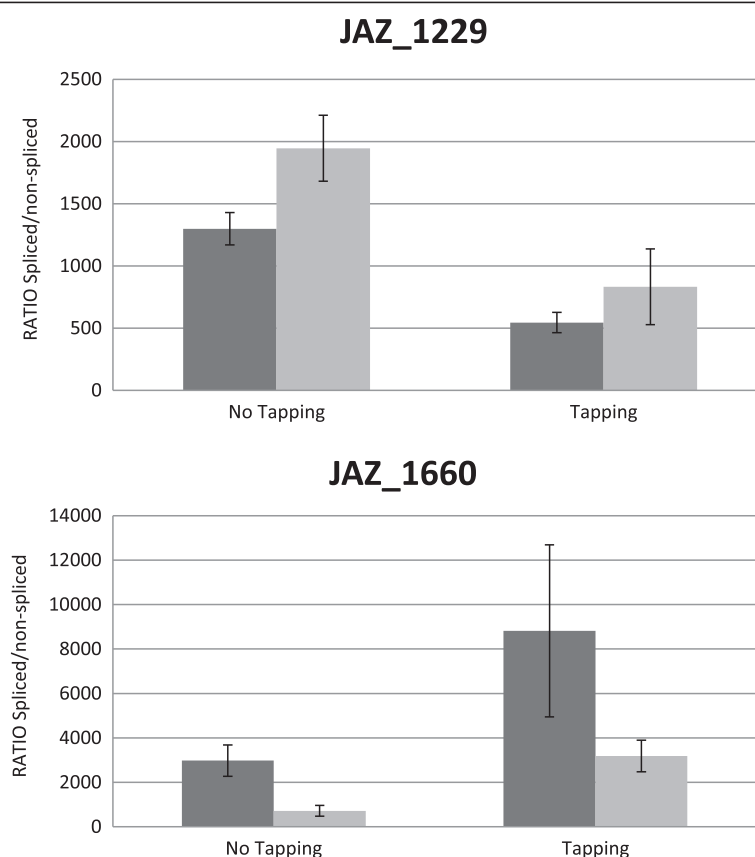


Figure 6 Regulation of alternative splicing by tapping and ethephon treatment in PB260 clone. The data presented correspond to the ΔC_t spliced form/ ΔC_t unspliced form ratio in latex. Ratios were studied under four conditions, untapped and tapped (left and right bars, respectively), stimulated or not by 2.5% ethephon (grey and black bars, respectively). The data presented correspond to averages of 3 independent biological replicates.

grouped *MYC_771*, *MYC_424*, *JAZ_1405* and *JAZ_17062* in the same cluster. Given that the transcripts of *MYC_424* and *JAZ_17062* were not significantly regulated by jasmonate, we focused on the transcriptional regulation of *JAZ_1405* by *MYC_771* and *MYC_94937* which, in response to JA, displayed the same expression profile. To that end, we cloned 2 versions of the *JAZ_1405* promoter (Figure 7A): a short sequence (*pJAZ_1405*(-267)) which contained 267 bp upstream of the ATG and which contained a G-box, known to be regulated by MYC factors [16] and a longer sequence (*pJAZ_1405* (-955)) with a size of 955 bp upstream of the ATG and which contained 2 G-boxes. The GFP reporter gene under the control of promoters *pJAZ_1405* (-267) or *pJAZ_1405* (-955) was co-transformed with 35S::HbMYC_771 or 35S::HbMYC_94937 (Figure 7A,B). The results showed that the 2 MYCs had a transcriptional activity that was twice as strong with the *pJAZ_1405*(-955) promoter than with the *pJAZ_1405* (-267) promoter (Figure 7C).

Discussion

Laticifer cells display a transcript pattern for genes acting in the jasmonate pathway that differs from the other tissues

To date, data regarding the jasmonate signalling pathway have been limited in latex plants to *Hevea*. Although a few genes acting in the jasmonate signalling pathway (*COI1*, *JAZ*, *MYC*) have been identified in *Hevea* [40-42], our study led to the identification of all the factors (6 *JAR*, 2 *COI*, 10 *JAZ*, 3 *MYC*, 1 *NINJA*, 1 *TPL*, 1 *MED25*). Recently, a sequence of *HbCOI1* was isolated from clone RRIM600 (EU136026) [40]. Our analyses enabled us to identify an allele of this gene in clone PB260, *HbCOI_2304*. *HbCOI_2304* displayed 95% identity with the sequence of RRIM600. So far, in *Hevea brasiliensis*, only one *JAZ* sequence has been identified (*HbJAZ1* : GQ369508) in clone RY-7-33-97 [41]. An analysis of the transcriptome of clone PB260 resulted in identification of the *HbJAZ_1660* sequence, which displayed 100% homology with *HbJAZ1*. In addition, the same laboratory isolated 2 transcription

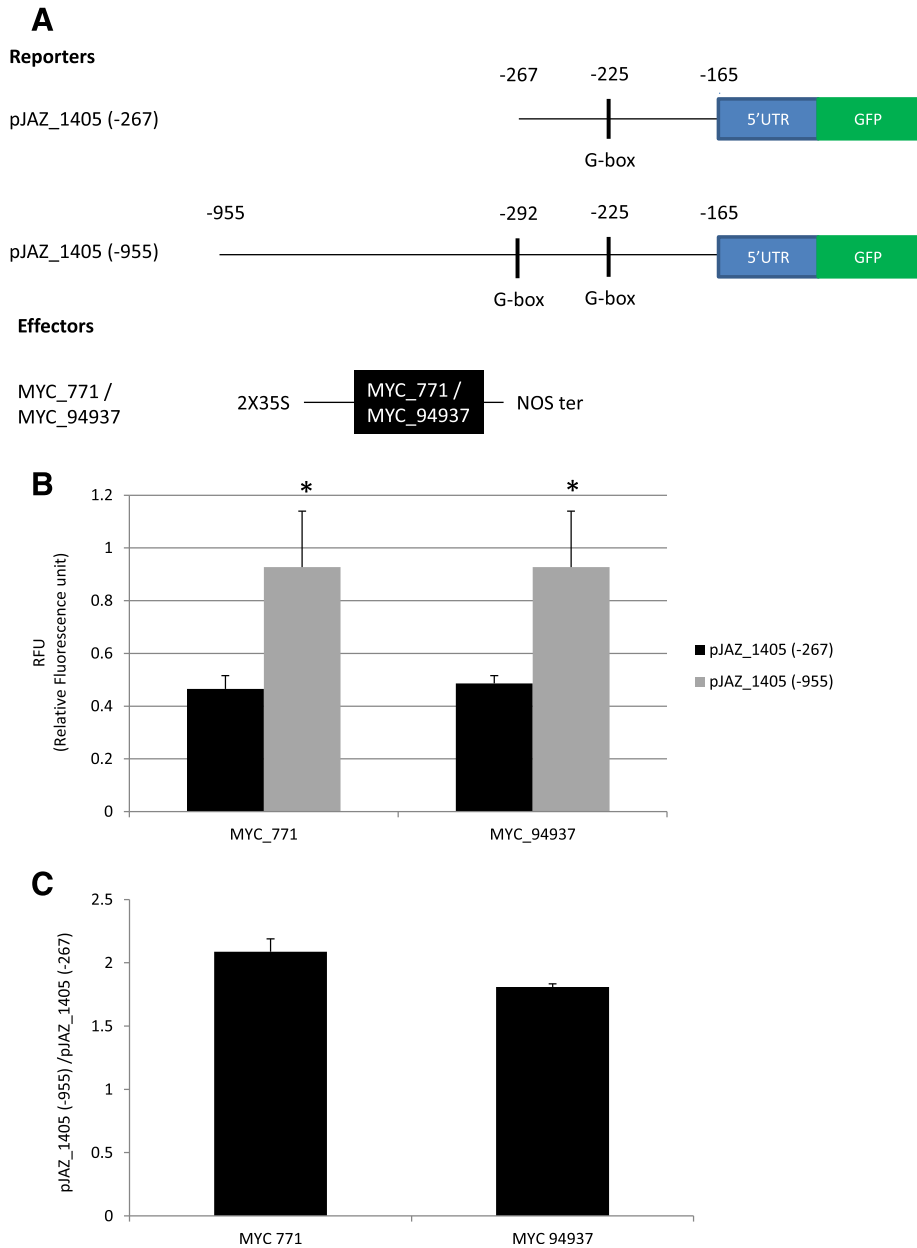


Figure 7 MYC_771 and MYC_94937 regulate transcription of JAZ_1405. Transient expression in a single cell system was used to assess the transcriptional effect of MYC_771 and MYC_94937 proteins on JAZ_1405. The fluorescence of the reporter gene was measured by flow cytometry upon co-transfection with a reporter construct (pJAZ_1405::GFP) and an effector construct (35S::HbMYC_771 or 35S::HbMYC94937). The basal fluorescence obtained in the assay transfected with the reporter construct and pJAZ_1405(-267) was taken as the reference (100% relative fluorescence). **(A)** Schematic representation of the constructs used for transient expression assays. The reporter construct consisted of the JAZ_1405 promoter and GFP coding sequence. The effector constructs expressed HbMYC_771 and HbMYC_94937 under the control of the CaMV 35S promoter. **(B)** The fluorescence of protoplasts co-transformed with pJAZ_1405::GFP and pMDC32 were used as reference (RFU = 1). Ratio of fluorescence from pJAZ_1405 (-267)::GFP or pJAZ_1405(-955)::GFP with HbMYC_771 or HbMYC_94937 on the reference fluorescence were displayed in RFU. Star indicate a significant difference of fluorescence obtained with the same MYC for long and short promoters. **(C)** Effect of MYC_771 and MYC_94937 on the JAZ_1405 promoter. The value with the short promoter was set at 1 and relative values are shown. Error bars indicate the standard deviation of results from 6 replicates.

factors, *HbMYC1* and *HbMYC2* (*HbMYC1*:GU434304 and *HbMYC2*: HM061097) in clone RY-7-33-97, which have been described as orthologous to *AtMYC2* [42]. An analysis of the transcriptome led to the identification of

these 2 genes. *MYC_424* displayed 99% identity with *HbMYC1*, like *MYC_771* orthologous to *HbMYC2*. The number of *COI* and *JAZ* gene models is similar in *Hevea*, rice, *Arabidopsis*, poplar, grapevine and *Brachypodium*,

but the number of *JARs* is larger in *Hevea* (6) than the one or two genes found in the other species. However, given the diversity of GH3s, it is highly likely that not all of them are involved in JA conjugation with Ile (Table 1).

Laticifers cells displayed an original signature of transcripts for the JA signalling pathway since they stood out from all the other tissues tested (Figure 1). Transcript analyses, obtained on PB260, confirmed the results published by Peng and coll. on RRIM 600 [40] showing that *HbCOI_2304* is preferentially expressed in latex. In addition, *HbCOI_3058* displayed a similar profile (Figure 1). In clone RY-7-33-97, Zhao and coll. showed that the transcripts of *HbMYC1* and *HbMYC2* were preferentially accumulated in latex [42]. Our results, obtained on clone PB 260 confirmed that tendency (Figures 1 and 2). However, our results did not confirm an abundance of *MYC_424* transcripts in response to JA observed in clone RY-7-33-97 [42] (Figure 2E). To date, no study has shown the potential implication of *JAZs* in the laticifer metabolism. An analysis of the *HbJAZ* expression profile showed that the transcripts of 5 *HbJAZs* were more highly accumulated in latex than in the other tissues. For instance, in addition to the transcripts of *COI*, *JAZ* and *MYC*, the transcripts of the *JAR_59958* and *JAR_20347* genes were highly accumulated in this tissue. All these results suggested that the jasmonate signalling pathway in laticifer cells involved specific actors. This confirmed the importance of studying this pathway in latex production but also in response to harvesting stress.

Harvesting stress can take both common and distinct wounding and dehydration pathways

Latex harvesting amounts to strong abiotic stress that can lead to a physiological syndrome such as TPD. A study of transcript abundance in response to wounding, dehydration, and to hormonal treatments, ethylene and jasmonate, helped to determine what types of abiotic stress and signalling pathways occurred during tapping. The jasmonate signalling pathway was regulated by all the types of stress tested, suggesting the involvement of that pathway in the response to each of the types of stress. Although the comparison was difficult, as the tissues and times tested were not exactly the same, our results

confirmed that the transcript abundance of *HbMYC1* and *HbMYC2*, corresponding to *MYC_424* and *MYC_771*, decreased after 6 h of MeJA treatment, then rose from 24 h onwards [42] (Figure 2E). The results suggested that the signalling pathways of abiotic and harvesting stress may activate the same genes. Indeed, *JAZ_863*, repressed by ethylene in the stem and by ethephon in bark, was at the same time induced by dehydration and tapping (Figures 2D and 3). In addition, *JAZ_29511* and *JAZ_14313* were induced by both wounding and tapping, while *JAZ_1229* was induced by dehydration and tapping (Figures 2 and 3). Despite that, the results in this study suggested that some independent wounding, dehydration and ethylene pathways were activated during latex harvesting. Indeed, *JAZ_2001*, *JAZ_14313* and *JAZ_26925* were induced by ethylene at 24 h, 8 h and 1 h respectively, but were repressed by ethephon in bark (Figures 2 and 3). In latex, the transcripts of *COI_2304*, *MYC_424* and *MYC_771* were accumulated in response to tapping (Figure 3), which confirmed the results in the literature [40,42]. Nevertheless, *JAZ* members are functionally redundant to some extent [17-19] and the diversity in their structures suggests specific roles for some of them. This hypothesis is corroborated by the diverse expression patterns displayed by *HbJAZ* in response to various types of abiotic stress.

HbMYC_771 and *MYC_94937* can regulate the *HbJAZ* genes containing a G-box type *cis*-element

In addition to negative regulation of *JAZs* on *MYCs*, it has been shown that there exists a positive regulation of *JAZ* transcription by *MYCs* via the G-box *cis*-element [16]. Hierarchical clustering grouped *MYC_771*, *MYC_424*, *JAZ_1405* and *JAZ_17062* in the same cluster (Figure 1). Given that the transcripts of *MYC_424* and *JAZ_17062* were not significantly regulated by jasmonate, we focused on the regulation of *JAZ_1405* by *MYC_771* and *MYC_94937* which, in response to JA, displayed the same expression profile (Figure 2). Transactivation experiments showed that the 2 *MYC* factors tested were able to regulate the transcription of *JAZ_1405* (Figure 7). It is highly likely that this activation of *JAZ_1405* by *MYC* factors occurs via the G-box present in the promoter of that gene. Among the promoters isolated from scaffold

Table 1 Number of identified members of *JAR*, *COI* and *JAZ* genes in the rubber tree, rice, *Arabidopsis*, poplar, grapevine and *Brachypodium*

Genes	Contigs	Number of genes (data from http://rice.plantbiology.msu.edu)				
		Rice	Arabidopsis	Poplar	Grapevine	Brachypodium
JAR	6	1	1	2	1	1
COI	2	2	1	2	1	2
JAZ	10	15	12	14	9	11

sequences, we were able to show that the promoters of *HbJAZ_1229* and *HbJAZ_19967* also possessed a G-box, suggesting transcriptional regulation of these 2 JAZs by MYCs. This last result may explain the grouping of *MYC_771* and *JAZ_1405* in the same cluster, but also a similar expression profile for *MYC_94937* and *JAZ_1405* in response to JA.

JAZ_1229 and JAZ_1660 are regulated by alternative splicing in the laticifer cells of tapped trees

The regulation of alternative splicing provides flexibility at transcriptome and proteome level, which helps plants adapt to their environment [66]. However, while the misregulation of alternative splicing has been associated with many human diseases, its biological relevance in plant systems is just beginning to be deciphered [67]. Many studies have shown the importance of AS and splicing factors (SF) in the response to abiotic stress (for review, [28]). With the alternative splicing of JAZ proteins, recent studies suggest that it makes it possible to establish a negative feedback loop to attenuate the response to JA in the event of overstimulation of the signalling pathway [26,27]. To date, this regulation of JAZs has never been linked to the response to abiotic stress. An analysis of the structure of *HbJAZs*, along with the sequencing results, confirmed the existence of alternative splicing of the *HbJAZ_1229* and *HbJAZ_1660* genes (Figure 5). An analysis of the exon/intron junction of these 2 *HbJAZs* showed that this splicing led to the introduction of a premature termination codon (PTC). In *Arabidopsis*, the introduction of that PTC leads to the production of a JAZ protein, whose jas domain is absent [26]. In general, the introduction of a PTC engages the transcript in the nonsense-mediated decay (NMD) pathway to degrade it [68,69]. According to the literature, these results suggest that truncated forms of *HbJAZ_1229* and *HbJAZ_1660* are dominant negative regulators [26,27,70]. For the first time, our results brought out a link between the regulation of JAZ alternative splicing and abiotic stress. Indeed, our results showed that alternative splicing of *JAZ_1229* and *JAZ_1660* could be regulated by tapping in latex. Curiously, that regulation was opposite for the transcripts of *JAZ_1229* and *JAZ_1660* (Figure 6). An analysis of the abundance of the 2 forms of transcripts of these 2 *HbJAZs* showed that the transcript abundance of the spliced form remained at least 500 times greater than the non-spliced form (Figure 6). This final observation tallied with what is generally observed in *Arabidopsis* since, although intron retention is the most common form of AS (~40%), many of these transcripts are not very abundant at all [71]. Taken together these results suggested a compensatory phenomenon and functional redundancy of *HbJAZ_1229* and *HbJAZ_1660*, which is all the more likely in that they were classed in the same cluster (Figure 1).

Genes acting in the JA pathway are expressed differentially in the latex of different *Hevea* clones

The results presented here show that the JA signalling pathway was regulated differently depending on the clones tested. Indeed, the negative JAZ regulators were overexpressed in PB260, while the transcripts of MYC factors and the JAR enzyme, which are positive regulators of the JA pathway, were accumulated preferentially in clones INC 53 and RRIM600 (Figure 4). These results suggested activation of the JA target genes in clones with a slow laticifer metabolism, more resistant to TPD.

The identification of signalling pathways involved in TPD resistance should make it possible to isolate the master regulators controlling resistance to this syndrome. The identification of JA transduction pathway members and knowledge of their expression pattern open up new ways of improving the TPD-tolerance of *Hevea* clones. The JA signalling pathway is very widely distributed and very highly conserved during evolution. However, a study on extra floral nectar (EFN) excretions by central American acacias revealed that resistance to biotic stress *via* the jasmonate pathway might be induced or constitutive in some phylogenetically very close species [72]. Thus, that study suggests that specific variability of the JA pathway may have some major consequences for resistance to stress.

Conclusion

The present study provides some molecular clues on how the Jasmonate pathway can be involved in harvesting stress in *Hevea brasiliensis* through (i) the specific expression pattern of jasmonate pathway actors in latex, (ii) their transcriptional regulation in response to harvesting stress, (iii) differential expression depending on the *Hevea* clone, (iv) their putative alternative splicing regulation in response to harvesting. A better understanding of transcriptional regulations during harvesting stress, along with the use of clonal diversity in response to such stress, are therefore a major challenge for improving resistance to stress and rubber production in *Hevea*.

Availability of supporting data

The authors confirm that all data underlying the findings are fully available without restriction. Character Matrix and phylogenetic tree have been deposited in treebase (ID: 16569) and data are available at the following URL: <http://purl.org/phylo/treebase/phyloWS/study/TB2:S16569>.

Additional files

Additional file 1: Sequences of the primers used for qPCR analysis.

Additional file 2: Comparison of Cp values, standard deviation and coefficient of variance for gene expression analysis by real-time RT-PCR of 11 housekeeping genes in abiotic stress conditions.

Additional file 3: Comparison of Cp values, standard deviation and coefficient of variance for gene expression analysis by real-time RT-PCR of 11 housekeeping genes in harvesting stress conditions.

Additional file 4: Sequences of the primers used for cloning of *MYC_771*, *MYC_94937* and *pJAZ_1405*.

Additional file 5: Amino acids sequence alignment of HbJAR1 with AtJAR1.

Additional file 6: Amino acids sequence alignment of HbCOI with AtCOI.

Additional file 7: Amino acids sequence alignment of HbJAZ with AtJAZ1.

Additional file 8: Amino acids sequence alignment of HbMYC with AtMYC2.

Additional file 9: Amino acids sequence alignment of HbNINJA with AtNINJA.

Additional file 10: Amino acids sequence alignment of HbTPL with AtTPL.

Additional file 11: Amino acids sequence alignment of HbMED25 with AtMED25.

Additional file 12: Analysis of variance of gene expression according to the tested tissues.

Additional file 13: Analysis of variance (ANOVA) to test effect of tapping, ethephon treatment, tissue and cross effects on the expression of the different gene of the JA signaling pathway.

Additional file 14: Analysis of variance of gene expression according to the tested clone.

Additional file 15: Analysis of variance (ANOVA) to test effect of tapping, ethephon treatment, tree age and cross effect on the ratio spliced/unspliced of *JAZ_1229* and *JAZ-1660*.

Competing interests

The authors declare that they have no competing interests.

Authors contributions

JP participated in the design of the study, performed the experiments. PP and MR carried out the total RNA isolation and cDNA synthesis from samples. FD carried out the transcriptional activity experiments in the single cell system. K supervised the plant culture experiments. CT supervised genome sequencing. JP, JL and PM drafted the manuscript. PM coordinated this research work. All the authors read and approved the final manuscript.

Acknowledgements

This work was supported by CIRAD. We are grateful to Prof. Songnian Hu from Beijing Institute of Genomics for access to the *Hevea* genome sequences. The authors also thank Peter Biggins for the English version of this manuscript.

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Received: 15 April 2014 Accepted: 19 November 2014

Published online: 02 December 2014

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doi:10.1186/s12870-014-0341-0

Cite this article as: Pirrello et al.: Transcriptional and post-transcriptional regulation of the jasmonate signalling pathway in response to abiotic and harvesting stress in *Hevea brasiliensis*. *BMC Plant Biology* 2014 **14**:341.

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